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(54) Title: IMMUNOASSAY METHOD AND REAGENT INVOLVING SUSPENDIBLE CARBON LABELLED BIOAFFINE PARTICLES			
<p>(57) Abstract</p> <p>An analysis method comprises establishing the presence of an analyte in a sample by forming a complex between the analyte and its bioaffine counterpart and a suspendible bioaffine reactant (R_1) which is labelled with carbon particles and incorporated in the complex in an amount related to the amount of analyte in the sample. The method is characterized in that a measurable part of the particles are able to settle. An immunoreagent labelled with particles and chosen from the group IgE, anti-IgE antibody, or allergen including IgE-reactive epitopes thereof characterized in that the particles are carbon particles.</p>			
<div style="display: flex; justify-content: space-around;"> <div style="text-align: center;"> <p>A general variant</p> <p>Sucking body</p> <p>Transport zone (Z_2)</p> <p>Control zone (Z_4)</p> <p>Transport zone (Z_2)</p> <p>Application zone (Z_1)</p> <p>Transport zone (Z_2)</p> <p>Application zone (Z_1)</p> </div> <div style="text-align: center;"> <p>An IgE-variant</p> <p>Anti-anime IgG</p> <p>Allergen or anti-IgE</p> <p>Sample plus labelled anti-IgE</p> </div> </div>			

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Immunoassay method and reagent involving suspendible carbon labeled bioaffine particles

5 TECHNICAL FIELD AND KNOWN TECHNIQUES

The analysis is concerned with determining the analyte content of a sample either qualitatively or quantitatively with the aid of its bioaffine counterpart, by forming a complex between the 10 counterpart and the analyte. In order to determine the analyte, there is used a bioaffine reactant (R_1) which is labelled with a group that can be shown analytically (= label) and which is incorporated in the complex. The analyte concentration of the sample can then be determined from the amount of complex-bound 15 and/or non-complex-bound labelled reactant (R_1' and R_1'' respectively).

The invention uses suspendible carbon particles as a label.

20 When the bioaffinity relates to antibodies and antigens/hapten, one speaks about immunoassays or immunochemical assaying. When the bioaffinity concerns complementary oligo/polynucleotides, one speaks about hybridization methods.

25 The invention is adapted particularly to immunoassays.

Many labels used in analysis processes based on biospecific affinity are known to the art. Particles are important in this context. According to known techniques, these particles will 30 primarily have colloidal dimensions - be in the form of sols (i.e. normally 1-200 nm and spherical and monodispersed). Well-known particulate label groups are metal particles, non-metal particles (e.g. SiO_2 , and carbon, latex and killed erythrocytes and bacteria). Particular mention can also be made to labels 35 that can be shown indirectly, such as individual members of bioaffine pairs, e.g. biotin or (strept)avidin, hapten or antibodies, class-specific determinants in antibodies, etc. The indirectly detectable labels are often determined or assayed with the aid of their bioaffine counterparts labeled with

labels that can be detected directly or indirectly.

Various methodologies are available in this regard depending on when, where and how an analysis is carried out and the material 5 to be analyzed. Examples of typical methods are:

- a. Heterogenous and homogenous methods. The terms heterogenous and homogenous describe whether or not labelled reactants (R_1') present in the complex are separated from non-complex-bound reactants (R_1'') prior to assaying labelled reactants.
10
- b. Competitive (inhibitions) and non-competitive methods. Competitive methods utilize analyte analogs which compete with analytes in binding to a deficiency of reactants that have biospecific affinity to both the analyte and the analyte analog. Analyte analogs may be analytes that are labelled with or bound to an insoluble phase (solid phase) in the analysis medium. Examples of non-competitive methods
15 are so-called sandwich methods in which the analyte is complexed between two or more reactants that exhibit biospecific affinity to mutually spaced epitopes on the analyte.
20
- c. Agglutination tests which involve clumping together of colloidal particles that are coated with a reactant which takes part in the aforementioned complex forming process.
25
- d. Immunohistochemical methods.
30
- e. Methods involving precipitation/insolubilizing processes using a reactant which selectively insolubilizes the complex bound or non-complex-bound labelled reactant, either via biospecific affinity (e.g. DASP), ion-bonding,
35 hydrophobic interactions, covalent bonding, etc.

The aforesaid methods have been carried out in more or less homogenous solutions, on test strips, porous matrices, in test tubes, microtiter wells, on flat surfaces, etc.

Known techniques relating to the use of carbon particles as labels and test strips for immunochemical assays with the aid of particle-labelled immunoreagents.

5

A. Waller et al, Laboratory Animal 11 (1977) 93-97, describes immunochemical assaying in which serum samples are treated with India Ink followed by contacting the sample with antigen from Encephalitozoon cuniculi. India Ink is

10 ink/drawing ink based on stably suspended carbon black (sol) (Hackhs Chemical Dictionary, 4th Ed., Ed. Julius Grant, McGraw-Hill Book Company, page 346).

B. EP 321,008 (HBT Holland Biotechnology) relates to an immunochemical method that uses colloidal, monodispersed non-metallic particles (carbon particles are mentioned) bound to an analyte or to the bioaffine counterpart of the analyte. The patent emphasizes that the particles shall be in the form of sols, meaning that particles which are able 15 to settle to form a sediment are not present in the particle preparations used. The patent makes particular mention to the production of sols by enclosing non-metallic colloidal primary particles in an inert hydrophilic envelope or by coupling reactants directly to the naked 20 particle. The patent mentions that sensitivity increases with increasing particle sizes, provided that the particle 25 preparations used are monodispersed particles in sol form.

C. Amerongen, et al (J. Biotechnol. 30 (1983) 185-189 and Clin. Chim. Acta 229 (1994) 67-75) relates to immunochemical assaying with the use of carbon particles in sol form as labels. The sol used is produced in accordance with EP 321,008. Neither Amerongen, et al, nor EP 321,008 discloses anything with regard to source, characteristics or 30 manufacture of the carbon particles that are said to be suitable for use. The technique using carbon particles is applied to test strips and quantitative analysis is effected with computer-based image analysis. As in the case 35 of EP 321,008, emphasis is made of the importance of the

particle preparations being in sol form.

D. Diverse. U.S. 4,318,707 (Syva) describes homogenous immunochemical assaying with the use of fluorescent-labelled reactants in combination with sound-adsorbing particles (e.g. sol) with bioaffine reactants bound thereto. GB 2,005,019 describes charcoal as an antigen carrier in heterogenous immunochemical assaying processes.

10 E. During the priority year the Swedish Patent Office has carried out an International Type Search. The report cites GB 2,239,314 that in general terms describes immunoassays employing as the labels carbon particles similar to the ones used in our experimental part, and EP 239,318 and WO 15 8911102 that discuss various types of particles among which charcoal is included.

OBJECTS OF THE INVENTION

20 The object of the present invention is to improve and simplify analysis methods of the kind defined in the introduction with the use of carbon particles as label groups.

FIGURE 1

25 This illustrates an embodiment of the invention (test strip) for determining total IgE with the aid of a sandwich test using solid-phase bound anti-IgE antibodies (R_2) bound in a detection zone (Z_1), labelled anti-IgE antibodies (R_1) and a control zone (Z_4) containing solid-phase bound anti-IgG antibodies (in our case anti-mouse IgG because R_1 is a mouse monoclonal). The sample application zone (Z_3) is located in the lower part of the strip and the reagent R_1 is mixed with sample prior to its application to the strip at zone Z_3 . Transport zones (Z_2' and Z_2'' and Z_2''') are located between the sample application zone (Z_3) and the detection zone (Z_1), between the detection zone (Z_1) and the control zone (Z_4) and between the control zone and a sucking body, respectively. That part of the strip which remains above the control zone may be understood as being a further transport zone (Z_2''''). The arrow indicates the

direction of flow in the strip. The reactants can be replaced as discussed below for different types of analytes. By replacing the solid-phase bound antibody R_2 for an antigen/hapten, the test will measure antigen/hapten-specific IgE (see the following examples).

THE INVENTION

The invention is based on the discovery that carbon particles of non-colloidal dimensions, preferably polydispersed and/or of irregular shape, function as labels and can even have a beneficial effect on the aforesaid analysis methods. Irregular particles have a greater surface/volume ratio per particle than spherical particles and thus give greater response or sensitivity to the analyses in which they are used as labels.

One aspect of the invention includes methods of the kind defined in the introduction. These methods generally include

- 20 i. contacting a sample that contains an analyte, possibly a pretreated sample/analyte, with a suspendible bioaffine reactant (R_1) labelled with carbon particles, under conditions which permit the formation of a bioaffine complex in which R_1 is present in an amount corresponding to a qualitative or quantitative measurement of the analyte concentration of the sample, whereafter
- 25 ii. the amount of particle-labelled reactant bound (R_1') or non-bound (R_1'') to the complex is determined qualitatively or quantitatively, wherein
- 30 iii. the determined quantity is taken as a measure of the presence or amount, respectively, of the amount of analyte in the sample.

35

The inventive method is characterized in that a measurable quantity of the carbon particles is so great that the particles (in the form of particle-labelled bioaffine reactant (R_1)) settle in an assay buffer at +4°C over a period of seven

calendar days. Whether or not a suspension fulfils this condition can be decided by visual determination after having allowed the suspension to stand under the aforesaid conditions.

5 According to the results disclosed in the experimental portion of this document, sensitivity increases with the amount of particles that can settle in accordance with the preceding paragraph. This means that the amount of particles that are able to settle is often > 0.1%, such as > 1%, preferably > 50%,
10 with an upper limit of 100% (all percentages being w/w percentages, alternatively measured in accordance with the method described under the heading "Sensitivity as a function of the amount of settleable particles" in the experimental part of this document).

15 In preparing the particles concerned, it is particularly preferred

- 20 a. that the carbon particles have a weighted mean particle size of > 0.05 μm , preferably in the range of 0.2-5 μm and more preferably in the range of 0.2-1 μm ; and
- 25 b. that > 1%, such as > 50%, with an upper limit of 100%, of the particles will lie in the range of 0.2-5 μm , preferably in the range of 0.2-1 μm .

The aforesaid sizes are measured with the aid of a Disk-Centrifuge Photosedimentometer using instruments from Brookhaven Corporation, U.S.A.

30 When measuring in accordance with "Flow-Field-Flow-Fractionation" (= FFF): (Jengthun Li, et al, Size Analysis of a Block Copolymer-Coated Polystyrene Latex, ACS Symposium Series No. 472, Particle Size Distribution 2, Chapter 16, pp. 246-262
35 (1991) American Chemical Society) corresponding preferred sizes are

- a. the top value of the particle distribution gives a diameter which is > 0.1 μm , preferably a diameter in the range of

0.2-5 μm and more preferably in the range of 0.2-1 μm ; and

b. > 1%, such as > 10% or > 50%, with an upper limit of 100% of the particles, is > 0.1 μm and preferably lies in the range of 0.2-5 μm , and more preferably in the range of 0.2-1 μm .

Suitable particle sizes and percentages of settleable particles will vary in accordance with the test method applied, analyte, affinity and specificity of the bioaffine reagent used. The main rule is that, compared to a higher analyte concentration, a lower analyte concentration will demand a greater portion of particles that are > 200 nm. Among other things, low-concentration analytes ($\leq 10^{-10}$ M) normally require particle preparations in which > 50% of the carbon particles lie in the range 0.2-1 μm (measured in accordance with FFF) (corresponding limits of particle sizes measured in accordance with "Disk Centrifuge Photodensedimentometer" are > 25% of the particles are greater than 0.2-1 μm). In the case of high-concentration analytes ($> 10^{-10}$ M), it is necessary to reduce the signal strength and to increase the quantity of reagent, which means, among other things, that the portion of particles > 0.2 μm is advantageously lowered to a level approaching the lowest percentages (alternatively one lowers the mean particle size (Disk Centrifuge Photosedimentometer) or the top mean value (FFF)). Analyte concentrations $> 10^{-7}$ M, these concentrations are normally measured by other means.

The subject matter of the preceding paragraph is related primarily to lateral immunoassay chromatography, although the person skilled in this art will be able to adapt the points discussed to suit other methods that employ biospecific affinity reactions, by reasoning in a corresponding manner. See also the experimental part.

35

Tests carried out hitherto have been effected with commercially available carbon-particle preparations in the form of primary aggregates of primary carbon nodules (primary particles) stably linked together, most likely covalently. The size of the

nodules varies from carbon quality to carbon quality, but is normally in the range of 5-250 nm, with preference for 20-100 nm. The number of primary nodules contained in each primary aggregate also varies, and is 4-200 with regard to the most 5 usable qualities, with preference to 6-75. By the term "stably linked together" is meant that the aggregates will not disintegrate under the conditions normally applicable to biospecific affinity reactions. The configuration of the primary aggregates may also vary from an elongated, branched 10 configuration to more compact structures.

The primary aggregates may, in turn, be secondarily aggregated to form larger units. When these secondary aggregates are stable against disintegration, they can be used in the 15 inventive analysis method provided that their sizes have no deleterious effect on the method applied.

A number of potentially usable carbon qualities (aggregates consisting of covalently bound primary nodules) which fulfil 20 the aforesaid definitions are commercially available from Degussa (Germany) and other manufacturers. Examples are carbon black, which is the collective name for furnace black, channel black, lamp black and acetylene black. The carbon content of these materials is usually from 85% and more, terminating at a 25 content immediately beneath 100% (99.999%) (w/w). The manufacturers have lowered the carbon content from 99-100% down to 85-99.9% (w/w), by oxidative treatment of carbon black, among others. Oxidatively-treated forms of the carbon particles concerned have been found particularly usable in the inventive 30 method, due to improved suspendibility when preparing the carbon-labelled reactant R₁.

The aforesaid carbon particles may be enclosed in polymeric envelopes and/or may carry bioaffine reactants or other groups 35 that are able to take part in hydrophobic interactions, ion-binding, dipole, dipole-binding, etc. One or more particles may be enclosed in one and the same polymeric envelope or casing.

Bioaffine reactants R₁ may be bound adsorptively directly to

the naked aggregates or via an envelope in accordance with the previous paragraph. In this latter case, the possibility of covalent bonding is obvious and covalent bonding directly to the naked aggregates is also potentially feasible. Bioaffine
5 reactants are best adsorbed on the carbon particles that are in suspended form.

According to preferred embodiments, the labelled bioaffine reactant is exogenous to the sample, and normally also
10 exogenous to the individual/organism/source from which the sample derives.

Examples of sample pretreatments are a) sample dilution, b)
sample concentration, c) analyte enrichment (e.g. removing
15 disturbing substances), d) pH-adjustment or adjustment by the use of other means, e) forming complexes between the analyte and a bioaffine counterpart, f) analyte modifications other than complex formation according to e), and so on. Points e) and f) above imply that the analyte in steps i-iii refers to an
20 analyte that is a modified version of the analyte in the original sample. All pretreatment processes are carried out so that the amount of analyte in the original sample and the amount of modified analyte in the pretreated sample will be unequivocally related to one another.

25 Analysis conditions, such as temperature, concentration, pH, sequence of additions of reactants and selection of reactants to be added are analogous with the conditions previously applied in respect of other labels used in assays which employ
30 biospecific affinity reactions (immunochemical assaying, hybridizing methods, etc.). The assay medium is normally water, optionally mixed with water-miscible solvents. In the case of immunochemical reactions, the temperature is in the range of 0-40°C and the pH in the range of 4-10. In the case of reactions
35 that involve hybridization between complementary oligo/poly-nucleotides, the temperature will preferably lie in the range of 0-100°C.

EMBODIMENTS PREFERRED IN PRACTICE**Heterogenous methodology**

5 The most practical embodiments of the invention employ heterogenous methodology, i.e. a technique in which labelled reactant R_1' included in the complex is separated physically from labelled reactant R_1'' which is not included in the complex, prior to assaying one or both of said reactants. In
10 the majority of cases, said reactants are separated by using a phase (matrix) which is insoluble in the assay medium and which has a bioaffine reactant bound thereto, to achieve selective partition of the complex-bound labelled reactant to the matrix before separation takes place.

15 The insoluble phase (the matrix) used in heterogenous variants of the invention may have the form of a strip, plate, porous continuous matrix (monolith), hydrophilic particles, preferably porous particles, membranes, diaphragms, etc.

20 The prerequisites for separating, assaying and correlating analytes as a function of labelled reactants are the same as those applicable in known techniques, with the exception of being adapted to the fact that according to the invention, the
25 label group consists of particles.

Porous matrices - test strips and other immunochromatographic techniques

30 The heterogenous method according to the invention considered most practical at present utilizes porous matrices, either in the form of particle-packed beds or in the form of monoliths (self-coherent matrices).

35 Monolith-type matrices may be given the form of test strips or membranes which include a detection zone (Z_1) for the detection of formed complexes. The test strip or membrane will also normally include one or more zones (e.g. Z_1' and Z_1'') for the transportation of sample, reactant (e.g. R_1), washing solution,

etc., to and/or from respective detection zones, and a sample application zone (Z_1), and optionally further zones Z_4 , Z_5 , etc. The transport zone Z_2 lies between the sample application zone and the detection zone.

5

The zones need not necessarily be comprised of the same material. For instance, it is often practical to choose material with regard to the function of the zone. See below.

10 The detection zone Z_1 contains a bioaffine reactant (R_2) which is stably bound to the matrix and which is capable of capturing labelled reactant R_1 in a quantity which is a function of the analyte content of the sample. When analyte is present in the sample, the detection zone will blacken. Blackening of the
15 detection zone can be shown both quantitatively and qualitatively, in several ways, preferably visually although perhaps more preferably, and more reliably, through the medium of computer-based picture or image analysis in accordance with Amerongen, et al (J. Biotechnol. 30 (1983), pp. 185-189, and
20 Clin. Chim. Acta 229 (1994), pp. 67-75).

In the simplest cases,

a. the stably bound reactant R_2 and the labelled reactant R_1 are both directed against the analyte (sandwich test); or
25
b. the bioaffine reactant R_2 stably anchored to the detection zone is directed against epitopes present in both analyte and reactant R_1 (R_1 is an analyte analog) (competitive
30 test).

The reference to R_2 being stably anchored in the detection zone is meant to imply that R_2 will not be eluted by the various assay-buffers that pass through. The reactant R_2 is anchored in
35 a conventional manner, i.e. covalently bound or bound via adsorption (normally via hydrophobic forces).

The simplest heterogenous variant of the invention using a porous matrix in the form of a packed particle bed or in the

form of a monolith, has only one detection zone Z_1 , this zone also functioning as a sample application zone. Reagent (e.g. R_1) and washing solution are applied directly to the detection zone and leave the matrix immediately after having passed through said zone.

In a simple further development, the detection zone is in contact with one or two transport zones (Z_2' and Z_2'') for transportation to and from the detection zone respectively. Z_2'' is preferably highly absorbent so as to facilitate liquid transportation. When a transport zone Z_2' is present, the sample application zone Z_1 will be juxtaposed thereto.

The reactant R_1 can be contacted with the detection zone in a number of different ways. It is generally preferred to bring reactant R_1 into contact (incubated) with analyte so that they are able to wander into the detection zone (Z_1) together. One variant is to pre-incubate/mix the sample with reactant R_1 prior to its application to the sample application zone. Reactant R_1 may also be pre-dispensed in the transport zone Z_2' or in the sample application zone Z_1 , so that R_1 is transported together with analyte to the detection zone Z_1 . Conceivable alternatives include pre-dispensing the reactant R_1 in the detection zone Z_1 or in a zone which is separated physically from the hitherto mentioned zones, or is applied directly to the detection zone Z_1 subsequent to having bound the analyte thereto.

In some variants of the invention, the biospecific reactants R_2 , which is stably linked to the detection zone is not capable of binding directly to the analyte. It may then be necessary to preincubate, e.g. the sample or the detection zone Z_1 or the transportation zone Z_2' , with a bifunctional affinity reagent that is capable of creating a link between the analyte and R_2 .

The sample can be transported from different zones to the detection zone by capillary forces when the matrix is initially dry to a substantial extent. Alternatively, a suction body may be included in the vicinity of the transport zone Z_2' .

(transportation from detection zone). Transportation can also be effected with the aid of an electric field. When the sample application zone is located at a higher level than the detection zone, transportation can be effected with the aid of 5 hydrostatic pressure, particularly in the case of porous matrices in the form of membranes.

Washing of the detection zone after applying different reagents will often require a surplus liquid absorption capacity in a 10 position downstream of the detection zone. This can be achieved by increasing the volume of the transport zone Z_2 , or by placing a highly absorbent matrix in liquid-flow contact therewith.

15 The sample application zone Z_1 often has the form of a porous matrix which possesses good water retention properties and, at the same time, is well capable of releasing water to the transport zone Z_2 during the analysis process. The matrix may be sufficiently hydrophilic to permit the transportation of 20 liquid. The matrix shall have a porosity which will enable the carbon particles to wander from the sample application zone to the detection zone. By way of a guide line, it can be said that the pore size shall be greater than the size of the carbon particles. The pore size will preferably lie in the range of 25 0.4-100 μm , in order to obtain an acceptable function of hydrophilic matrices.

At the priority date of this publication, we were successful in testing porous nylon matrices and porous nitrocellulose 30 matrices in the transport zone. Nylon, nitrocellulose and cellulose (paper) were used in the detection zone. Nylon and cellulose have been used for reactants R_2 that are anchored covalently, and nitrocellulose has been used for reactants R_2 that have been anchored adsorptively. The material used in the 35 sample application zone was the same as the material used in the transport zone, optionally in combination with a cellulose filter and/or fiberglass filter as a liquid reservoir.

Particle size, porosity and hydrophobicity can be optimized

with respect to one another by simple experimentation in a ready manner. See the experimental part.

The most preferred embodiment geometrically in the case of 5 porous matrices is a test strip in which the sample application zone, transport zone and detection zone are placed laterally after one another, with the possibility of liquid transportation therebetween. In the case of embodiments in 10 which surplus labelled reactant R_1 is transported from the detection zone Z_1 into a transport zone Z_2'' , it may be advantageous to place downstream of the detection zone a control zone (Z_4), through which R_1 is forced to pass. When the 15 control zone contains a bioaffine reactant which binds to R_1 , the control zone will be blackened in response to negative samples (and also in response to positive samples), therewith providing a function control.

The matrix will preferably be white in colour, even though pale nuances of other colours which provide good contrasts with 20 black carbon particles are acceptable.

For a more detailed description of the construction of test strips and the choice of material connected therewith, reference is made, inter alia, to WO 8808534 (Unilever), U.S. 25 5,120,643 (Abbott Labs), U.S. 4,954,452 (Abbott Labs), Amerongen, et al (J. Biotechnol. 30 (1983), pp. 185-189, Clin. Chim. Acta 229 (1994), pp. 67-75), and others, which also deal with the use of label groups in the form of colloidal particles.

30

Particularly preferred heterogenous techniques

According to heterogenous sandwich techniques, R_1 and R_2 may be 35 antibodies that are directed against physically spaced epitopes on the analyte, which is an antigen or a hapten having at least two epitopes. This technique is used to determine primarily high molecular weight, bio-organic molecules, such as proteins, e.g. Ig of a given class (IgA, IgD, IgE, IgG and IgM). In another sandwich variant, one of R_1 and R_2 is an antigen/hapten

and the other is an antibody directed against a class-specific determinant (IgA, IgD, IgE, IgG and IgM). This latter variant measures antigen-specific antibodies of a given class (analyte). When R₁ is an anti-IgE antibody and R₂ is the 5 antigen, there is obtained a PAST-type RAST-test (allergy test; PAST = particle Allergy Sorbent Test). Sandwich techniques can provide a very high degree of sensitivity and are therefore particularly suited for low-concentration analytes.

10 In the most preferred competitive technique, R₁ is an analyte analog and R₂ is an antibody directed against both the analyte and the analyte analog. This technique is primarily directed towards low-molecular bio-organic molecules and can be used to assay high molecular weight proteins, such as IgE.

15 The inventive method has been developed primarily for assaying serum/plasma levels levels of total-IgE and allergen-specific IgE for the purpose of allergy diagnosis, and corresponding methods in relation to other Ig classes. At the priority date 20 of this publication, the ranges of IgE concentration in this type of use was 10⁻¹⁰ - 10⁻⁷ M total IgE/l serum and 10⁻¹² - 10⁻⁹ M allergen-specific IgE/l serum respectively (i.e. both total IgE and allergen-specific IgE are low-concentration analytes).

25 The term antibody as used here includes intact antibodies and antigen-binding antibody fragments. The term antigen as used here includes the antigen as such, hapten and antigen fragments that exhibit antibody-binding activity.

30 OTHER ASPECTS OF THE INVENTION

Another aspect of the invention is an immunoreagent which is labelled with particles and chosen from the group comprising IgE, anti-IgE antibody and allergen, including IgE-binding 35 epitopes thereof. This aspect is characterized in that the particles are carbon particles. In the case of preferred embodiments, the particles are defined in the same way as those used in the inventive method.

THE FIELD OF USE OF THE INVENTION

The invention can be used in clinical diagnosis (including therapy monitoring processes), to which the aforesaid particle ranges are directly applicable and in many cases necessary.

5 Typical samples are: Cerebro Spinal fluid (CSF), saliva, lachrymal fluid, urine, blood (plasma and serum), etc.

Diagnostic areas of particular interest are assays in which an analyte is assayed in order to follow up a treatment (monitoring) and to diagnose various inflammatory conditions, such as asthma, and allergy-related conditions, and prolonged alcohol over-consumption. Examples of relevant analytes are antigen/allergen-specific antibodies of different classes (particularly IgA, IgD, IgE, IgG and IgM), total levels of respective Ig classes, eosinophilic cationic protein (ECP), eosinophilic protein X (EPX), myeloperoxidase (MPO), human neutrophilic lipocalin (HNL), lysozyme, tryptase, interleukins (IL-5, IL-6, IL-8), hyaluronic acid (HA), osteocalcin, etc. For alcohol over-consumption carbohydrate deficient transferrins (CDT; particularly asialotransferrin and disialotransferrin) are important analytes. This does not mean, of course, that the inventive method cannot also be applied in the diagnosis of other illnesses, such as cancer, hormonal disorders, etc.

25 The invention can also be applied in conjunction with quality controls in the production of substances which exhibit biospecific affinity. In this latter context the concentrations are often much higher and completely different requirements are placed on sensitivity and specificity. The demand placed on the carbon particles is also correspondingly lower, meaning that suspendibility will often suffice. This applies, inter alia, to IgE, anti-IgE antibodies, allergen and allergen epitopes (= haptens deriving from allergens) which bind to antibodies (particularly to IgE) and other earlier mentioned analytes and antibodies directed thereagainst.

The invention can also be applied in environmental analyses, for instance for determining the presence of allergens in

ambient air. Allergens normally derive from pollen, mites, cats, etc., and often have a protein structure.

The invention has primarily been developed for measuring low-concentration analytes by which are meant analytes that are measured in a range extending wholly or partly below 10^{-10} , i.e. concentrations within $1-10 \times 10^{-10}$ belong to the low-concentration range. This ranges refer to the concentrations of the analyte as it is present in its natural environment (CSF, plasma, serum, whole blood, urine, lachrymal fluid, saliva etc.).

The invention will now be illustrated in more detail with the aid of a number of non-limiting examples. The invention is defined in the following Claims.

EXPERIMENTAL PART

THE PREPARATION OF LABELLED REAGENTS

Carbon suspension (mother liquor): About 500 mg of carbon particles were suspended in 50 ml of a 5 mM borate buffer at pH 8.5 and sonicated for thirty minutes. Prior to the adsorption of protein on the carbon particles, the suspension was diluted to 1 mg/ml and sonicated for a further three minutes. The carbon quality used will be apparent from the following description of respective tests.

Labelling of anti-IgE antibodies with carbon particles (aggregate): A determined number of micrograms of mother liquor according to the above ($X \mu\text{g}$ carbon; see respective applications) and 50 μg of anti-IgE antibodies (in 5 mM borate buffer, pH 8.5) were diluted with 5 mM borate buffer pH of 8.5 to a final volume of 1 ml. The solution was allowed to stand for three hours at room temperature, after which 10 mg of bovine serum albumin (BSA) were added for each milliliter and the mixture was allowed to incubate for a further thirty minutes. The mixture was then washed three times with 0.1 M borate buffer, pH 8.5, containing 1% BSA and 0.05% NaN_3 , with

intermediate centrifugation at 16,000 x g. Upon completion of the last wash, the sediment was diluted with borate buffer (0.1 M, pH 8.5, 1% BSA, 0.05% NaN₃) to a carbon content of 500 µg/ml.

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The quantities used were adapted so that the anti-IgE antibody concentration was 25-50 µg/ml in the final solution. The centrifugation times varied with different types of carbons.

10 **Labelling of casein with carbon particles (aggregate):** Mother liquor containing 250 µg carbon (sp 100, Degussa) and 100 µg casein was diluted to a final volume of 1 ml with 5 mM borate buffer, pH 8.5. Labelling of anti-IgE antibodies was analogous in other respects.

15

MANUFACTURE OF TEST STRIPS

Test strip 1: A nitrocellulose membrane (Schleicher and Schuell, Germany, 8 µm) was laid against a stiff pre-gummed polyester sheet. An anti-IgE antibody solution was then sprayed onto the sheet in a line (detection zone) parallel with one side of the sheet, and an anti-Fc mouse IgG antibody solution was sprayed onto the sheet in a line (control zone) parallel with and well-spaced from the first line. The membrane was allowed to dry and was then clipped into strips measuring 0.8 cm x 4 cm (with the antibody lines parallel with the short ends of the strips). A filter paper (Whatman cellulose 17 CHr, England, measuring 0.8 cm x 0.8 cm (sample application zone)) was placed at the short end of the strip (at the bottom of the strip) on the same side as the detection zone. A suction filter (Whatman cellulose 17 CHr, 2 cm x 2 cm) was placed at the other end of the strip (at the top of the strip). The control zone was omitted in certain tests. The precise measurements varied slightly from case to case.

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Test strip 2: Test strip 2 was analogous with test strip 1 with the exception that the detection zone was comprised of a strip of cellulose paper (3 mm in width) to which mite allergen was covalently bound (CNBr coupling), the strip was clipped

from a mite disc (d1 allergen disc), Kabi Pharmacia Diagnostics AB, Sweden). The nitrocellulose membrane had been cut-off in the position of the detection zone, and the mite strip gummed to the test strip with opposite ends overlapping (membrane on 5 top and mite strip beneath in the lower part of the detection zone (the part facing in the flow direction)) and vice versa in the upper part.

Test strip 3: A nylon membrane. Anti-IgE antibodies, birch 10 allergens or casein were bound covalently in the detection zone.

STANDARD PROTOCOL FOR LATERAL IMMUNOASSAY CHROMATOGRAPHY

15 Total IgE: 30-80 µl of a mixture of three parts standard solution/sample (varying contents of IgE in 6% BSA, 50 mM phosphate buffer, pH 7.5, 0.05% NaN₃) and part of a carbon suspension (about 0.1 µg carbon/µl) onto which anti-IgE antibodies had been adsorbed in accordance with the aforesgoing 20 (X = 390 µg carbon, sp 100 (Degussa)) was applied to the sample application zone on test strip 1 and transportation towards the detection zone was initiated. A fibre glass filter wetted in buffer (1% BSA in 0.1 M borate, pH 89.5, 0.05% NaN₃) was then placed in the sample application zone. A dark band could be 25 detected in the detection zone for IgE-containing samples within ten minutes. The signal was easily distinguishable from results obtained with samples that did not contain IgE. Blackening of the strip was measured with a video camera (Kappa, CF 8/1) mounted on a zoom microscope (Askania MZM-1) 30 and calculated in a picture analysis program.

Mite-specific IgE: 38 µl of a positive human serum containing 125 kU/l of mite-specific IgE or blank sample (normal human serum < 0.35 kU IgE/l) were mixed with 12 µl of suspended 35 carbon particles (2.3 µg carbon) on whose surfaces anti-IgE had been adsorbed in accordance with the aforesgoing (X = 250 µg carbon Pr 150T (Degussa)) and applied directly to the sample application zone on the test strip 2. A wash was then initiated, by applying 40 µl 0.1 M borate (containing 1% BSA,

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0.05% NaN₃, pH 8.5) followed by a further wash with 20 µl of the same borate buffer, which now also contained 0.1% Tween® 20. The positive sample resulted in clear blacking of the detection zone. No blacking was obtained with the blank sample.

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Casein-specific IgE: 45 µl of a positive human serum (RAST positive with regard to casein) was diluted with normal human serum to 20 kU/l of casein-specific IgE per litre or of a blank sample (human serum < 0.35 kU IgE/l) and mixed with 7.5 µl (5 µg carbon) of a suspension of carbon particles into which casein had been adsorbed in accordance with the foregoing, and then applied directly to the sample application zone on test strip 1. Subsequent to absorption of the sample into the zone, a wash was initiated in the same manner as that for total IgE. The positive sample resulted in unequivocal blackening of the detection zone. No blackening was observed in the case of the blank sample.

SENSITIVITY AS A FUNCTION OF THE AMOUNT OF SETTLEABLE PARTICLES
20 (AGGREGATES)

Aggregate labelled anti-IgE antibodies: Tests were carried out with ¹²⁵-anti-IgE antibodies labelled in accordance with the Chloramine T-method. The labelled antibody was then adsorbed on 25 carbon particles (sp 100 (Degussa, Germany) in accordance with the above method. Mother liquors were stored in a refrigerator at +4°C and all of the particles were found to have settled after two calendar days. Suspensions containing adsorbed antibodies were centrifuged at different G numbers, to obtain 30 fractions of different sizes (in the form of pellets and supernatants).

Percentage of settleable particles in respective fractions: Respective pellets were re-suspended and allowed to settle at 35 room temperature over a period of eleven calendar days. Supernatants were also allowed to settle under the same conditions. The radioactivity of the sediments was determined and taken as a measurement of the portion/percentage of settleable particles in respective fractions. The results are

set forth in **Table 1** below.

Ability to wander in a membrane (pore size 8 μm): Re-suspended sediment, supernatant, or non-centrifuged suspension was placed in the sample application zone of test strip 1 and allowed to wander towards the detection zone. A certain degree of blackening remained in the sample application zone, due to the fact that some carbon particles fastened in the zone. The extent of the radioactivity remaining in the sample application zone was taken as a measurement of the particles that were unable to wander. The results are set forth in **Table 1**.

Signal strength as a function of carbon aggregate sizes: The procedure applied was in accordance with standard protocol for lateral immunoassay chromatography. The samples used were IgE-standards (0.5-50 kU IgE/l, 6% BSA, 50 mM phosphate buffer, pH 7.5, 0.05% NaN₃). Because the low measuring range is the range of most interest when testing allergen-specific IgE, the signal strength was compared at 0.5 kU IgE/l. The signal strength was converted to relative measurement values (signal strength:mean signal). The fractions were divided into three groups: supernatants (3 in number), non-centrifuged suspensions (2 in number) and pellets, sediment (3 in number). The results are set forth in **Table 1**.

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Table 1. Signal strength as a function of aggregate size measured in terms of settleability. Carbon quality: sp 100 (Degussa).

30	Fraction	% sediment	start zone	Relative
		11 cal. days	%-carbon remaining	signal strength for 0.5 kU IgE/l
	Supernatant	17	3.6	0.59
35	Non-fractionated	44	7.1	1.20
	Pellets	52	14.4	1.20

In summary, it was found that:

1. The fraction (pellets) having the smallest particle sizes (supernatants) gave a weak signal with low IgE levels.
2. Non-centrifuged suspensions which settled (> 95%) when stored in an assay buffer (0.1 M borate buffer, 1% BSA, 0.05% NaN₃, pH 8.5) at +4°C to +8°C for two calendar days gave a better test than the fractions which were enriched with regard to particles having lower settling tendencies.
- 10 3. Fractions containing large particles (pellets, sediment) were equally as sensitive as non-fractionated suspensions of the same carbon sorts, due to the fact that the major part of the increase in sediment remained in the sample application zone.

15

SIGNAL STRENGTH DEPENDENCY ON DIFFERENT CARBON QUALITIES

About twenty different types of carbon black (Degussa), particularly channel black and furnace black and certain particular black-types were tested with regard to the adsorption of IgG (¹²⁵I-anti-IgE antibody) and their function in tests (lateral immunoassay chromatography). We found it impossible to produce suspensions from several of the carbon blacks used. This problem was solved in many cases, by sonicating carbon mixed with antibodies, although certain types of carbon still produced suspensions which were less ideal. The method for total-IgE in accordance with the foregoing was used as standard protocol for lateral immunoassay chromatography. The samples used were IgE standards, containing 0.5-125 kU IgE/l.

All carbon sorts tested gave a positive signal in the region over 5 kU IgE/l, although only a small percentage gave a strong signal in the low measuring range. The degree of blackening was correlated to the IgE level for all carbon sorts. The control zone was blackened with all the carbon sorts studied.

Because the lower part of the IgE range is of most interest when testing allergen-specific IgE, the signal strength was

compared at 0.5-1 kU/l with a plurality of carbon sorts. The measured values were converted to relative values (signal strength:mean signal). The carbon sorts were divided into three groups with 6-7 carbon sorts in each group, depending on the function of the low IgE values in the assay. The tendency of the carbon particles on which anti-IgE antibodies had been adsorbed to settle (+4°C for a month) from the suspension was taken as a measurement of aggregate size. A suspension from which all carbon particles had settled to provide a complete sediment was given the value of 4+, whereas a suspension which resulted in no sediment was given the value 0+. The results are set forth in Table II below.

Grouping	Settling tendency	Relative assay response (low IgE)
Best	3.4+	1.7
Mean	2.8+	1.1
Worst	2.0+	0.5

20 **In summary, we found that:**

1. All carbon sorts adsorbed anti-IgE antibodies (^{125}I -anti-IgE antibodies).
- 25 2. In the case of lateral immunoassay chromatography, the carbon sorts exhibited clearly different performances - a higher settling tendency resulted in increased test sensitivity.

30 **Comparison tests at sp 100 and sp 4 (Degussa)**

The test protocol was the same as that used for total IgE. Two analysis series were carried out with the same amount of carbon of two different carbon sorts (sp 4 and sp 100 respectively). The IgE contents of the samples were 1, 5, 20 and 100 kU/l. Standard curves for respective carbon sorts show that sp 100 gives a higher signal than sp 4 in the case of low IgE concentrations, but that on a molar basis sp 4 carried more

reagent, resulting in a higher signal at higher concentrations. The tendency of larger particles/aggregates to produce stronger signals was also observed when corresponding comparisons were made on different membranes. Sp 100 produces with:

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0.5 kU/l IgE a signal which is 2.6 times higher than the signal produced with sp 4.

1.0 kU/l IgE a signal which is 2.4 times higher than the signal produced with sp 4.

10

5.0 kU/l IgE a signal which is 1.7 times higher than the signal produced with sp 4.

20 kU/l IgE a signal which is 1.3 times higher than the signal produced with sp 4.

15 DETERMINING THE SIZE OF CARBON PARTICLES (PRIMARY AGGREGATES)

The diameter of aggregate sizes for sp 100 and sp 4 (Degussa) were determined by:

20

1. Flow-Field-Flow-Fractionation (= FFF): (Jengthun Li, et al, Size Analysis of a Block Copolymer-Coated Polystyrene Latex, ACS Symposium Series No. 472, Particle Size Distribution 2, Chapter 16, pp. 247-262 (1991) American Chemical Society). The diameter of carbon sp 100 and sp 4 (Degussa) was determined. The peak value of the particle distribution gives a diameter of 280 nm for sp 100 and a diameter of 140 nm for sp 4. The results are set forth in **Table III** below:

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Particle size	< 100 nm	100-200 nm	> 200 nm
sp 100	5%	18%	77%
sp 4	18%	48%	34%

Disk Centrifuge Photosedimentometer (= DCP): The measuring processes were carried out with instruments from Brookhaven Corp., U.S.A. The results are set forth in **Table IV** below:

25

Particle size	< 100 nm	100-200 nm	> 200 nm
sp 100	30	28%	42%
sp 4	70	28%	2%

5 Weighted mean values are 200 nm for sp 100 and 85 nm for sp 4.

Carbon particle sizes were measured indirectly in terms of their ability to settle, and directly by FFF and DCP. FFF and DCP are analysis methods which focus on different aspects of 10 particle geometry. The results obtained (Tables III and IV) illustrate differences that are a consequence of the particles being irregular and non-spherical with complicated geometry.

EXPERIMENTAL RESULTS OBTAINED DURING THE PRIORITY YEAR.

15 During the priority year, the performance of the test strip mode of the invention has been improved. Our recent findings support that, at least with respect to sandwich heterogenous techniques, it is preferred to use as immune reagents antibodies in the form of fragments lacking the Fc-portion.
 20 This is at least applicable to labelled reactant R₁. With respect to the order of addition, the sequential mode is preferred, i.e. a mode which is arranged so that the analyte/sample and the labelled reactant R₁ is transported through at least a part of the transportation zone (Z₂) in
 25 this order. Preferentially this means that they are added separately to the strip, for instance at different times to the application zones (example 1 below, sample before R₁) or more preferably at different locations in the transportation zone between the application zone and the detection zone (example 2
 30 below, R₁ to an early segment of the transportation zone Z₂ after addition of sample to the application zone).

1. Total IgE, sequential mode.

30 µl IgE in buffer (different IgE concentrations in 6% BSA, 50
 35 mM phosphate buffer pH 7.5, 0.05 % NaN₃) was applied on one end (the liquid application zone) of a polyester supported nitro-cellulose membrane (8 µm, Whatman, UK) with the dimensions 5x30 mm. The membrane carried a glued thin polyester strip about 7 mm downstream from the application zone to prevent spreading of

the applied liquid on the membrane surface.

Anti-IgE (Pharmacia Diagnostics, Uppsala, Sweden) had previously been adsorbed in a thin line (1x5 mm) 2 cm from the application end (detection zone). A sucking cellulose pad (5x15 mm Whatman cellulose 17 Chr) was placed at the top of the nitro-cellulose membrane, downstream the adsorbed anti-IgE.

When the sample had migrated into the membrane, 30 µl of a washing buffer (1% BSA, 0.05% bovine gamma globulin, 0.1 M borate buffer pH 8.5, 0.9% NaCl, 10% sucrose, 0.05% NaN₃) was applied on the application zone. After this washing step, 30 µl of carbon-labelled anti-IgE fab'2 (anti-IgE fab'2 labelled in analogy with previous examples from 6 µg sp 100 + 0.6 µg anti-IgE fab'2 in washing buffer) was placed on the application zone. After adsorption of this suspension a final washing step was performed by applying a cellulose pad, containing 0.1 ml of washing buffer (see above), on the liquid application zone. 10 minutes after the sample application, a black band became gradually visible on the reaction zone 2 cm downstream from the liquid application zone. The intensity of the band was related to the amount of IgE in the applied sample. The intensity was measured with a CCD camera mounted on a zoom microscope and calculated with a computer image program.

25

Table V

	IgE kU/l	CCD units
30	0	364
	0.13	600
	0.25	771
	0.5	1189
	2.0	3131
	5.0	5444
35	20	8211
	50	8790
	200	9314
	500	9736
	2000	10341

2. Allergen (casein) specific IgE in human plasma, sequential mode.

30 µl patient sample (diluted in a negative plasma, see table below) was applied on one end (the liquid application zone) of
5 a polyester supported nitro-cellulose membrane (8 µm, Whatman, UK) with the dimensions 5x30 mm. The application was done via a filtering membrane (Cytosep 1661, Gelman, USA) mounted on top of the nitro-cellulose membrane. The membrane carried two glued thin polyester strips glued to the nitro-cellulose membrane
10 about 7 and 13 mm, respectively, downstream from the application zone to prevent spreading of the applied liquid on the membrane surface.

Casein had previously been adsorbed in a thin line (1x5 mm,
15 detection zone) 2 cm from the application end. A sucking cellulose pad (5x15 mm Whatman cellulose 17 Chr) was placed at the top of the nitro-cellulose membrane, downstream the adsorbed casein.

20 When the sample had migrated into the membrane, 30 µl of a washing buffer (1 % BSA, 0.05 % bovine gamma globulin, 0.1 M phosphate buffer pH 7.5, 0.9 % NaCl, 10 % sucrose, 0.05 % NaN₃) was applied on the application zone. After this washing step, 30 µl of carbon-labelled anti-IgE fab'2 (labelling in analogy
25 with previous examples from 6 µg sp 100 + 0.6 µg anti-IgE fab'2 in washing buffer) was placed on the zone between the polyester strips immediately followed by application of 30 µl of washing buffer on the application zone. 15 minutes after sample application, a black band became gradually visible in the
30 detection zone located 2 cm downstream from the liquid application zone. The intensity of the band was related to the amount of casein-specific IgE in applied sample. The intensity was measured with a CCD camera mounted on a zoom microscope and calculated with a computer image program.

Table VI

	Casein specific IgE kU _A /l	CCD units
	Negative plasma	320
5	0.55	622
	1.1	957
	4,5	2064
	45	6573

10 The results presented in tables V and VI illustrate that concentrations at least as low as 10^{-12} can be measured.

15 The order of additions secured that the analyte had passed the application segment for carbon-labelled anti-IgE fab'2 (R_1) before addition of carbon-labelled anti-IgE fab'2 (R_1) was added. It further prevented transport of carbon-labelled anti-IgE fab'2 (R_1) into the application zone.

CLAIMS

1. An analysis method wherein the presence of an analyte in a sample is established by forming a complex between the analyte and its bioaffine counterpart and a suspendible bioaffine reactant (R_1) which is labelled with carbon particles and incorporated in the complex in an amount related to the amount of analyte in the sample, characterized in that a measurable percentage of the particles are able to settle from the suspension.
2. A method according to Claim 1, characterized in that the particles have the form of stable primary particle aggregates.
- 15 3. A method according to any one of Claims 1-2, characterized in that each aggregate contains between 4-200 primary particles.
- 20 4. A method according to any one of Claims 2-3, characterized in that the primary particles have a size in the range of 5-250 nm, preferably in the range of 20-100 nm.
- 25 5. A method according to any one of Claims 1-4, characterized in that the carbon particles are carbon black.
6. A method according to any one of Claims 1-5, characterized in that the carbon particles are chosen from the group furnace black, channel black, lamp black and acetylene black.
- 30 7. A method according to any one of Claims 1-6, characterized in that the carbon particles are treated oxidatively prior to binding the particles to R_1 .
- 35 8. A method according to any one of Claims 1-7, characterized in that the carbon content of the particles is 85-99.99% (w/w).
9. A method according to any one of Claims 1-8, characterized by using a porous matrix which includes a sample application zone (Z_1), a detection zone (Z_2) and a transport zone (Z_3).

located therebetween for transporting analyte and liquid to the detection zone (Z_1), wherein the method includes the steps of

- 1) applying the analyte to the sample application zone (Z_1) and allowing the analyte to wander to the detection zone
5 (Z_1), said detection zone containing a stably anchored bioaffine reactant R_1 , capable of binding the analyte; and thereafter
- 2) establishing the specific presence of analyte bound in the detection zone, with the aid of the reactant R_1 , in the
10 form of blackening said zone.

10. A method according to Claim 9, characterized in that the labelled reactant R_1 and the analyte are incubated with one another prior to wandering into the detection zone.

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11. A method according to any one of Claims 8-10, characterized in that R_1 is an antibody directed against the analyte, and R_2 is an antigen, or vice versa, and the analyte is an antigen-specific antibody.

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12. A method according to Claim 11, characterized in that R_1 is a class-specific antibody selected from the group anti-IgA, anti-IgD, anti-IgE, anti-IgG and anti-IgM antibody, particularly anti-IgE antibody.

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13. A method according to any one of Claims 8-9, characterized in that R_1 and R_2 are antibodies directed against physically spaced epitopes on the analyte; and in that the analyte is a multi-epitopic antigen, wherein preferably R_1 and R_2 are anti-
30 IgE antibodies and the analyte is IgE.

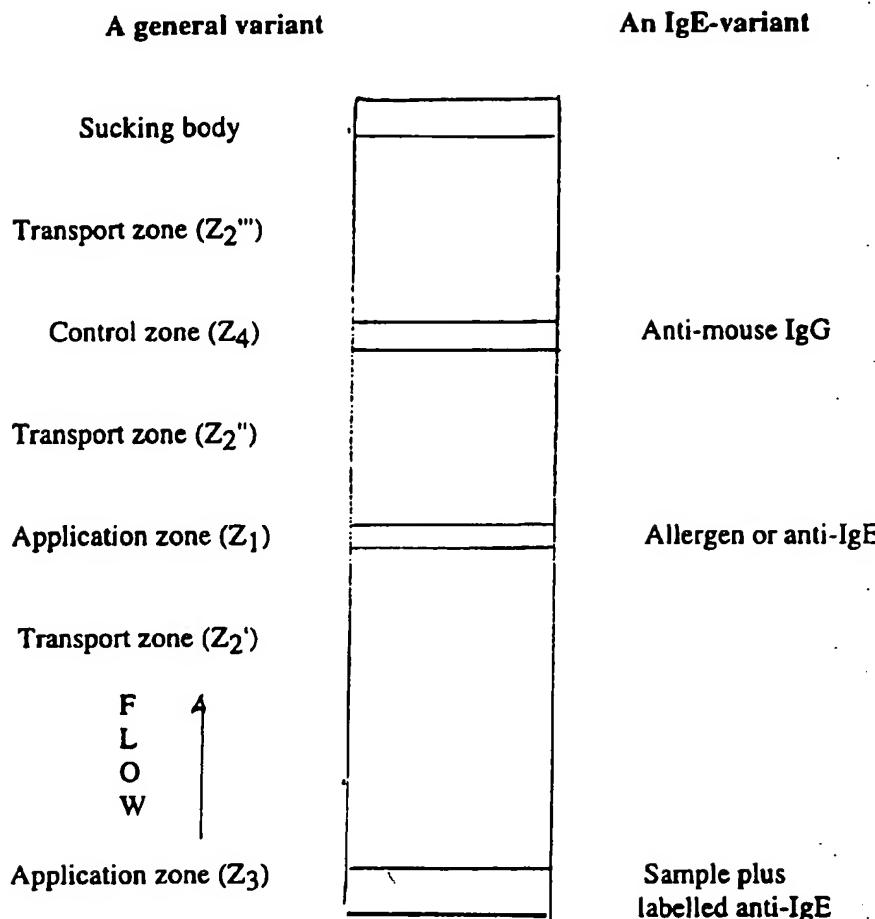
35

14. A method according to any one of Claims 8-12, characterized in that the porous matrix is continuous and has the form of a test strip on which the zones are arranged laterally.

15. An immunoreagent labelled with particles and chosen from the group IgE, anti-IgE antibody, or allergen including IgE-reactive epitopes thereof, characterized in that the particles are carbon particles.

16. An immunoreagent according to the preceding Claims,
characterized in that the particles are defined in the same way
as that defined in Claims 1-7.

1/1

FIGURE 1

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 96/00042

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: G01N 33/543, G01N 33/532

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GB 2239314 A (PRINCETON BIOMEDITECH CORPORATION), 26 June 1991 (26.06.91) --	1,2,5,6,8-16
X	WO 8911102 A1 (ANGENICS, INC.), 16 November 1989 (16.11.89), see page 20, lines 20-26 and page 31, lines 22-31 --	1,9,15,16
X	EP 0239318 A1 (FISHER SCIENTIFIC COMPANY), 30 Sept 1987 (30.09.87), see page 5, lines 3-19 --	1,9,15,16
A	EP 0321008 A1 (H.B.T. HOLLAND BIOTECHNOLOGY B.V.), 21 June 1989 (21.06.89) --	1,2,5,6,8-16

 Further documents are listed in the continuation of Box C. See patent family annex.

- * Special categories of cited documents
- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
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- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 May 1996	107 -05- 1996
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. + 46 8 666 02 86	Authorized officer Carl-Olof Gustafsson Telephone No. + 46 8 782 25 00

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 96/00042

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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International application No.

PCT/SE 96/00042

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